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VOL. 22, SEC. B. SEPTEMBER, 1944 NUMBER 5

THE ALKALOIDS OF *LYCOPODIUM* SPECIES

VI. *LYCOPODIUM CLAVATUM* L.[1]

BY LÉO MARION[2] AND RICHARD H. F. MANSKE[3]

Abstract

Lycopodium clavatum L. of American origin has yielded nicotine, lycopodine, alkaloid L13, and two new, as yet uncharacterized alkaloids, referred to as alkaloids L18 and L19. The alkaloids, clavatine and clavatoxine, found in *L. clavatum* of European origin were absent and it is suggested that the two plants are specifically or varietally distinct.

Lycopodium clavatum L. of European origin has been chemically examined by Achmatowicz and Uzieblo (1) who recorded the isolation of lycopodine and two new alkaloids, clavatine and clavatoxine. Taxonomists have regarded the American *L. clavatum* L. as identical with the European plant and the authors confidently expected to isolate the above alkaloids from the plant of Canadian origin. Lycopodine was indeed isolated as it was from a number of other Canadian species, but neither clavatine nor clavatoxine could be isolated, and furthermore it is certain that neither is present in more than infinitesimal amounts. The authors are inclined to believe that, in spite of the taxonomic similarity, the two plants are specifically or at least varietally distinct. This premise is considerably substantiated not only by the above negative evidence but by the fact that three alkaloids not isolated by Achmatowicz and Uzieblo were obtained from the plant under investigation, albeit in only very small amounts. One of these proved to be identical with alkaloid L13 already isolated from *L. tristachyum* (4) and from *L. obscurum* (3). The formula ($C_{16}H_{25}ON$) has been substantiated by an analysis of the pure base, which has now been obtained in the crystalline condition. The remaining two alkaloids appear to be new and are referred to as alkaloids L18 and L19. The empirical formula of the former appears to be $C_{11}H_{15}ON$, which is based on an analysis of the picrate. The latter was obtained in amounts insufficient for complete analysis.

In addition to the above bases a small amount of nicotine was also isolated.

[1] *Manuscript received March 28, 1944.*

Contribution from the Division of Chemistry, National Research Laboratories, Ottawa, Canada. Issued as N.R.C. No. 1208.

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Experimental

The plant material was collected in August on the north shore of the Ottawa River, some fifty miles upstream from Ottawa. Dried and ground, it weighed 1515 gm. It was worked up as already described (2). The total content of alkaloids was 2.2 gm. (0.145%).

Nicotine

The crude alkaloid was slowly distilled *in vacuo*, the distillation flask being heated in an air-bath. The fraction boiling up to 160° C. (2 mm.) was collected and the undistilled residue kept separately. The distillate when redistilled yielded the following fractions: Fr. I, b.p. 85 to 110° (2 mm.) and Fr. II, b.p. 150 to 160° (2 mm.).

Fraction I was combined with a similar fraction obtained from another lot of plant and was refractionated. A small fraction was obtained which boiled at 85 to 95° C. (2mm.) and the rest boiled at 110° (2 mm.). The lower boiling oily base was dissolved in methanol and added to a methanolic solution of picric acid. A lemon-yellow picrate separated which, after recrystallization from boiling methanol, melted at 223°* and in admixture with an authentic sample of nicotine dipicrate (m.p. 226°) melted at 224°. The base was recovered from the picrate and redistilled (b.p. 75 to 85° (2 mm.), wt. 39.8 mg.). It was dissolved in aqueous acetone and added to a hot solution of picrolonic acid (132.5 mg.) in aqueous acetone. The picrolonate which crystallized was filtered and recrystallized from a little acetone. It melted at 228° either alone or in admixture with an authentic sample of nicotine dipicrolonate.

Alkaloid L18

The oily base, boiling at 110° (2 mm.), obtained by refractionation of Fraction I was converted to the picrate in hot methanolic solution. A picrate separated as an oil which, with a great deal of difficulty, was finally induced to crystallize. It consisted of a mixture from which by repeated fractional crystallization from methanol a picrate was obtained which melted at 195° C. after sintering at 192°. Found: C, 49.35, 49.25; H, 5.43, 5.44; N, 13.80, 13.51%. Calc. for $C_{17}H_{22}O_8N_4$: C, 49.76; H, 5.37; N, 13.66%.

Lycopodine

The fraction, b.p. 150 to 160° C. (2 mm.), weighed 1.1 gm. It crystallized on standing and was recrystallized from a little absolute ether from which it separated as well formed colourless prisms. After one more crystallization from absolute ether it melted at 116° either alone or in admixture with lycopodine. The mother liquor was evaporated to dryness, the residue dissolved in acetone and the solution neutralized with 60% perchloric acid. A crystalline perchlorate separated immediately which was filtered and recrystallized from boiling water. It proved to be that of lycopodine.

* *All melting points are corrected.*

Alkaloid L13

The undistilled residue left after distillation of the crude base was redissolved in dilute (1 : 1) hydrochloric acid and the solution filtered through charcoal. The filtrate was basified with ammonia and the precipitated base collected in ether. After drying and evaporation of the solvent a gummy residue was left weighing 1.1 gm. It was dissolved in a little absolute ether, the solution filtered to remove a little insoluble amorphous material and evaporated to dryness. The residual base was fractionated *in vacuo*. It yielded a fraction, b.p. 130° C. (0.02 mm.) and one, b.p. 160 to 162° (0.02 mm.). The first fraction (b.p. 130° (0.02 mm.)) was dissolved in warm absolute ether from which it crystallized on standing. After repeated recrystallization from absolute ether it was obtained as small colourless plates melting at 130°. Found: C, 77.52, 77.66; H, 10.18, 10.28; N, 5.84, 5.77%. Calc. for $C_{16}H_{25}ON$: C, 77.73; H, 10.12; N, 5.66%.

Some of this base was dissolved in ethyl acetate and the solution neutralized with 60% perchloric acid. A crystalline perchlorate gradually separated which, after repeated recrystallization from methanol–ethyl-acetate, melted at 270° C. In admixture with a sample of the perchlorate of alkaloid L13 (m.p. 274°), it melted at 271°. This isomer of lycopodine has already been isolated from *L. tristachyum* (4) and from *L. obscurum* (3) as its perchlorate. This is the first time that the authors have succeeded in obtaining the base in the crystalline condition.

Alkaloid L19

The fraction which boiled at 160 to 162° C. (0.02 mm.) crystallized on standing. It was now insoluble in ether and was recrystallized from boiling methanol from which it separated in colourless short needles melting at 231°. When mixed with annotinine it was all liquid at 190°. Found: C, 60.22; H, 4.25%. There was not enough material for a complete analysis.

The lycopodine consisted of about 60% of the total alkaloid content. The other bases were obtained in very small quantities only. There was an appreciable undistillable residue from which no crystalline alkaloid could be isolated either as base or perchlorate.

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STUDIES ON THE BACTERIAL OXIDATION OF 2,3-BUTANEDIOL AND RELATED COMPOUNDS[1]

By R. Y. Stanier[2] and Sybil B. Fratkin[3]

Abstract

Aerobacter aerogenes, *Aerobacillus polymyxa*, and *Aeromonas hydrophila*, representatives of the three genera characterized by a butanediol fermentation, can all oxidize 2,3-butanediol under aerobic conditions. The configuration of the 2,3-butanediol has considerable bearing on its decomposability: *Aerobacter aerogenes* is inactive on the *l*-isomer, but attacks both *meso*- and *d*-isomers; *Aeromonas hydrophila* attacks the *meso*-isomer but not the *l*- and probably not the *d*-isomer; *Aerobacillus polymyxa* can oxidize both *l*- and *meso*-2,3-butanediol, but the rate with the former is many times greater than with the latter. *Aerobacter aerogenes* oxidizes both 2,3-butanediol and acetoin to carbon dioxide and water, a large part of the substrate being simultaneously assimilated. The other two organisms oxidize 2,3-butanediol to acetoin, but can further oxidize the acetoin thus formed only very slowly, if at all. Both *Aerobacter aerogenes* and *Aerobacillus polymyxa* are unable to attack 1,3-butanediol, 2-methyl-1,2-propanediol and 1,2-ethanediol. However they can oxidize 1,2-propanediol to acetol.

The oxidation of 2,3-butanediol by *Acetobacter* species has been investigated by Kling (6), Visser't Hooft (10) and more recently by Fulmer, Underkofler, and Bantz (4). It has been shown that *Acetobacter suboxydans* can produce acetoin from *meso*- but not from *d*-butanediol*; the reaction appears to stop at the acetoin stage since yields approximating 95% of theory have been obtained (Fulmer, Underkofler, and Bantz). However, information on the oxidation of butanediol by other bacteria, and particularly by organisms that can produce this substance in the fermentation of carbohydrates, is extremely scanty. Walpole (11) found that *Aerobacter aerogenes* could produce acetoin when grown aerobically in a medium containing Witte peptone and butanediol; the yield was small, amounting to only 0.55 gm. from 8.0 gm. of butanediol. Werkman (12) subsequently made similar observations, but as he merely

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[2] *Bacteriologist, Industrial Utilization Investigations.*

[3] *Biochemist, Industrial Utilization Investigations.*

* *Except where otherwise stated, the expression "butanediol" refers in this paper to 2,3-butanediol.*

established the production of acetoin qualitatively by the extremely sensitive Voges–Proskauer reaction, there is no way of telling whether the acetoin was the final product of oxidation or an intermediate compound present in traces.

We have undertaken a study of the oxidation of butanediol and related compounds by representatives of the three genera—*Aerobacter*, *Aerobacillus*, and *Aeromonas*—which are characterized by the production of butanediol during carbohydrate dissimilation under anaerobic conditions.

Materials and Methods

The organisms used were *Aerobacter aerogenes*, Strain 199, from the collection of the Northern Regional Research Laboratory of the United States Department of Agriculture; *Aerobacillus polymyxa*, Strains NRC–10 and NRC–20 (4) from our own collection; and *Aeromonas hydrophila*, Strain Ps. 2, obtained from Dr. E. R. Hitchner of the University of Maine.

The substrates investigated were 2,3-butanediol, acetoin, and diacetyl. Three different samples of butanediol, prepared by recovery from fermentations, were available: the *l*-isomer produced by *Aerobacillus polymyxa*, the mixture of *d*- and *meso*-isomers produced by *Aerobacter aerogenes*, and the mixture of *l*- and *meso*-isomers produced by *Aeromonas hydrophila*. The diacetyl was prepared by redistillation of the Eastman product, and the acetoin was made by Dr. A. C. Neish of this laboratory.

All experiments were performed manometrically using the Warburg apparatus. Runs were carried out at 30° C. in an atmosphere of air with 2.0 ml. of cell suspension and 0.2 ml. of substrate. In order to obtain cell material, the organisms were grown for 16 to 24 hr. in a liquid medium (0.5% glucose, 0.5% peptone), centrifuged, washed once, and resuspended in *M*/50 phosphate buffer (pH 6.7) or in tap water. Particularly in the case of *Aerobacillus polymyxa* young cultures must be used; cells from cultures more than 24 hr. old are inactive, probably owing to the deleterious effect of acid produced from the glucose.

Results

The Oxidation of 2,3-Butanediol by Aerobacter aerogenes

Since the observations of Walpole (11) and Werkman (12) indicated that acetoin was produced by the action of *Aerobacter* species on butanediol, experiments were started on the assumption that the oxidation proceeded

only to this stage. However, the addition of 40 μmoles of the *d–meso* mixture of butanediol to a suspension of *A. aerogenes* caused a rapid oxygen uptake far in excess of the 448 μlitres required for oxidation according to the equation:

$$CH_3 . CHOH . CHOH . CH_3 + \tfrac{1}{2}O_2 \longrightarrow CH_3 . CO . CHOH . CH_3 + H_2O. \qquad (1)$$

Hence it was apparent that *A. aerogenes* could carry the oxidation well beyond the acetoin stage. In all subsequent experiments much smaller amounts of substrate (of the order of 5 μmoles) were used.

With limiting amounts of butanediol, a characteristic curve for oxygen consumption is obtained (Fig. 1, Curves 3*A* and 3*B*). After mixing cells and

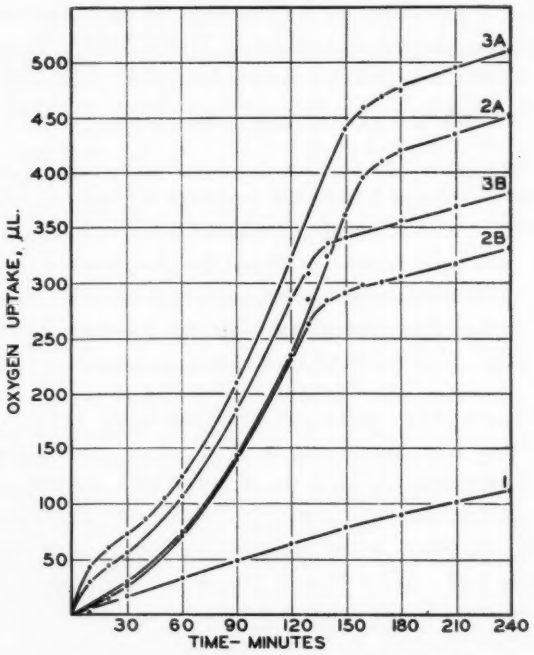


FIG. 1. *Oxygen uptake by Aerobacter aerogenes in the absence of substrate (Curve 1) and in the presence of 5.0 and 3.3 μmoles of acetoin (Curves 2A and 2B respectively) and of 5.0 and 3.3 μmoles of the d- and meso-mixture of 2,3-butanediol (Curves 3A and 3B respectively).*

substrate, there is at first a small but rapid oxygen uptake which falls off abruptly after 10 to 15 min. to a rate little higher than that of the autorespiration. The rate then gradually increases, ultimately reaching a steady level (if sufficient substrate has been used) which is maintained to the point of substrate exhaustion, when there is another sharp break to a rate approaching

that of the autorespiration. The height of the initial rise in oxygen uptake is approximately proportional to the amount of butanediol present (see Fig. 2),

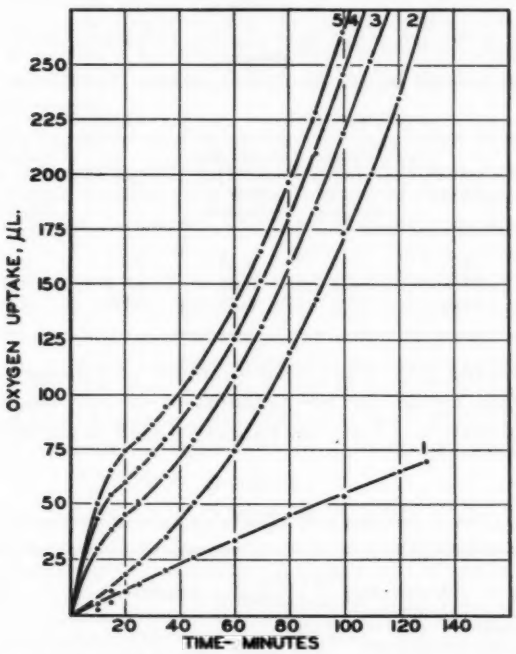


FIG. 2. *Oxygen uptake by Aerobacter aerogenes during the initial stages of butanediol and acetoin oxidation. Data taken from the same experiment as shown in Fig. 1, plotted on an enlarged scale to demonstrate the first phase of butanediol oxidation. Curve 1, autorespiration; Curve 2, 3.33 µmoles of acetoin; Curve 3, 3.33 µmoles of butanediol; Curve 4, 5.0 µmoles of butanediol; Curve 5, 6.67 µmoles of butanediol.*

while in the subsequent period of gradually increasing rate, the curves with different amounts of substrate parallel each other extremely closely, although running at the different absolute levels established by the initial rise. These facts suggest that the oxidation proceeds in two steps with markedly different initial rates. We interpret these two steps as being (i) a rapid oxidation of butanediol to acetoin followed by (ii) the further oxidation of the acetoin thus formed, a reaction for which the cells are initially unadapted as shown by the gradually increasing rate of oxygen uptake. There are a number of different facts that support this interpretation. First, the oxygen uptake with acetoin shows no trace of a sharp initial rise, but parallels exactly the oxygen uptake with butanediol during the subsequent stages (see Fig. 1, Curves 2*A* and 2*B*). Second, carbon dioxide production is negligible in the first stage of butanediol oxidation. Third, the total amount of oxygen consumed at the first break in the curve for oxygen consumption approximates the theoretical

for oxidation of butanediol to acetoin. Data illustrating this last point are set forth in Table I.

TABLE I

OXYGEN UPTAKE AT THE END OF THE FIRST STAGE OF BUTANEDIOL OXIDATION BY *Aerobacter aerogenes*

2,3-Butanediol, μmoles	Uncorrected			Corrected for autorespiration		
	O_2 uptake, μlitres	O_2 uptake, μlitres per μmole of substrate	O_2 uptake, μmoles per μmole of substrate	O_2 uptake, μlitres	O_2 uptake, μlitres per μmole of substrate	O_2 uptake, μmoles per μmole of substrate
3.33	38.0	11.5	0.51	32.0	9.7	0.43
5.0	53.5	10.7	0.48	47.5	9.5	0.42
6.67	68.0	10.2	0.45	62.0	9.3	0.41

Table II presents data drawn from a number of different experiments on the total oxygen uptake for the oxidation of varying amounts of *d–meso* butanediol and acetoin. The results are given both in uncorrected form and

TABLE II

OXYGEN UPTAKE BY *A. aerogenes* WITH VARIOUS AMOUNTS OF BUTANEDIOL AND ACETOIN

Substrate	Amount, μmoles	Theoretical O_2 uptake for complete oxidation, μlitres	Theoretical O_2 uptake for complete oxidation, μlitres per μmole of substrate	Uncorrected		Corrected for autorespiration	
				O_2 uptake, μlitres	O_2 uptake, μlitres per μmole of substrate	O_2 uptake, μlitres	O_2 uptake, μlitres per μmole of substrate
Butanediol	3.33	411	123.2	336	101.0	261	78.2
Butanediol	5.0	616	123.2	469	93.8	384	76.8
Butanediol	5.0	616	123.2	523	104.6	408	81.6
Butanediol	5.0	616	123.2	464	92.8	391	78.2
Butanediol	6.67	822	123.2	633	94.9	528	79.2
Butanediol	6.67	822	123.2	642	96.3	527	79.0
Acetoin	3.33	373	112.0	301	90.3	216	64.8
Acetoin	5.0	560	112.0	420	84.0	330	66.0
Acetoin	6.67	746	112.0	603	90.5	457	68.4

corrected for autorespiration. A number of investigators—Barker (1), Clifton and Logan (2), Doudoroff (3), van Niel (9)—have pointed out that when a substrate is being rapidly oxidized the autorespiration is almost totally suppressed and should not be taken into account in making calculations. Particularly van Niel (9) has deprecated the common custom of presenting "corrected" data for manometric experiments without including the figure for autorespiration. In the present instance, however, a careful inspection of the data forces one to the conclusion that the autorespiration is by no means

a negligible factor. Uncorrected figures for oxygen consumption with butanediol and acetoin show very marked divergences in the uptake per mole of substrate, whereas the corrected results are substantially constant in this respect. It must be remembered that the oxidation of acetoin is slow in the initial stages, and hence is not strictly comparable with the oxidations studied by the above-mentioned workers, which proceeded for the most part at a rapid and uniform rate.

Averaging the corrected oxygen uptakes for butanediol we obtain a figure of 78.8 μlitres per μmole of substrate oxidized. This is equivalent to 3.5 moles of oxygen per mole of butanediol, instead of the expected 5.5 moles required for complete oxidation. The average oxygen uptake for acetoin is 66.4 μlitres per μmole of substrate, or 3 moles of oxygen per mole of acetoin instead of the expected 5. Since the oxygen required for the oxidation of butanediol to acetoin is 0.5 moles per mole, it is apparent that this reaction goes to completion, while the oxidation of acetoin, whether secondarily formed or present as the primary substrate, results in an oxygen uptake that is only 60% of theoretical. Studies on carbon dioxide production during the oxidation of butanediol and acetoin (see Table III) show that in both cases the carbon dioxide produced is equivalent to 2 moles per mole of substrate oxidized, or 50% of theoretical.

TABLE III

OXYGEN UPTAKE AND CARBON DIOXIDE PRODUCTION (BOTH CORRECTED FOR AUTORESPIRATION) FOR THE OXIDATION OF 6.67 μMOLES OF BUTANEDIOL AND ACETOIN BY *A. aerogenes*

Substrate	Total O_2 uptake, μlitres	O_2 uptake, μlitres per μmole of substrate	O_2 uptake, moles per mole of substrate	Total CO_2 production, μlitres	CO_2 production, μlitres per μmole of substrate	CO_2 production, moles per mole of substrate
Butanediol	504	75.6	3.38	310	46.5	2.08
Acetoin	450	67.5	3.01	295	44.3	1.98

We interpret these large discrepancies as being due to oxidative assimilation of a portion of the substrate, a phenomenon that has been firmly established for many micro-organisms by Giesberger (5), Barker (1), Clifton and Logan (2), Doudoroff (3), and others (see van Niel (8)). On the basis of this assumption, the equations for the oxidation of butanediol and acetoin may be completed as follows:

$$CH_3 . CHOH . CHOH . CH_3 + 3.5\ O_2 \longrightarrow 2(CH_2O) + 2CO_2 + 3H_2O \quad (2)$$

$$CH_3 . CO . CHOH . CH_3 + 3\ O_2 \longrightarrow 2(CH_2O) + 2CO_2 + 2H_2O \quad (3)$$

The formula (CH_2O) is used to denote the assimilatory product, whose exact nature is not known, although on the basis of many experimental data (van Niel (8)), it appears to have the empirical composition of a carbohydrate.

A. aerogenes is also capable of oxidizing diacetyl; the rate is approximately the same as that for acetoin, but owing to the volatility of diacetyl and its reactivity with the concentrated alkali used in the Warburg vessels to absorb carbon dioxide, we have been unable to obtain good figures for total oxygen consumption.

A. aerogenes is almost totally inactive on *l*-butanediol, the corrected oxygen uptake from 5.0 μmoles of this compound being only 8 μlitres. Further confirmation of this inability to attack the *l*-isomer was provided by the oxygen uptake with the *l*- and *meso*-mixture of butanediol produced by *Aeromonas hydrophila*. This material has an optical rotation of −0.97, indicating a content of *meso*-butanediol of approximately 92.5%. The corrected oxygen uptake from 5.0 μmoles was 354 μlitres, instead of the 392 μlitres to be expected on the basis of Equation (2) had all the substrate been oxidizable; i.e., 90.3% of the possible total, which checks reasonably well with the *meso*-butanediol content calculated on the basis of optical rotation.

The Oxidation of 2,3-Butanediol by Aerobacillus polymyxa

Fig. 3 shows the oxygen uptake by *Aerobacillus polymyxa* acting on 25

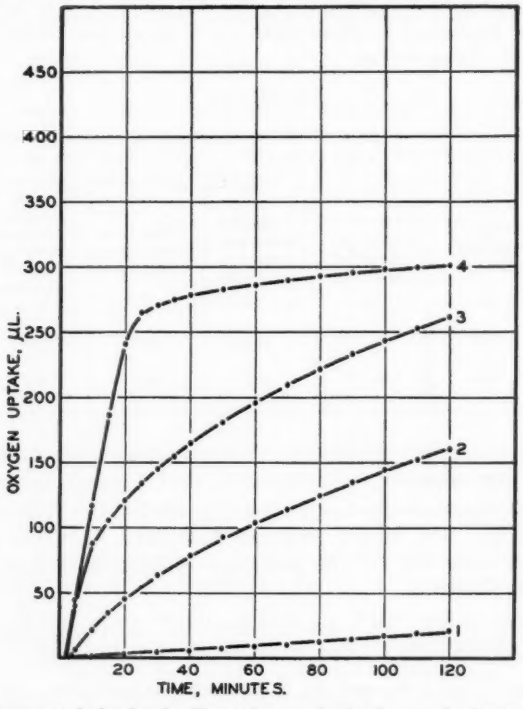


FIG. 3. *Oxygen uptake by Aerobacillus polymyxa in the absence of substrate (Curve 1) and in the presence of 50 μmoles of the d- and meso-mixture of butanediol (Curve 2), 50 μmoles of the l- and meso-mixture of butanediol (Curve 3), and 25 μmoles of l-butanediol (Curve 4).*

μmoles of *l*-butanediol (Curve 4) and 50 μmoles of the *d–meso* and *l–meso* mixtures (Curves 2 and 3 respectively). The *l*-butanediol is rapidly and completely oxidized to acetoin, the oxygen uptake at the point where the curve breaks being 270 μlitres as against a theoretical of 280 μlitres. The *d–meso* mixture on the other hand is attacked very slowly, at a rate approximately a tenth of that for the *l*-isomer. The *l–meso* mixture provokes a rapid preliminary oxygen uptake, doubtless owing to the oxidation of the *l*-component, and the rate then falls off to approximately that of the *d–meso* mixture. The difference between the curves for the *d–meso* and *l–meso* mixtures shows that *d*-butanediol, if attacked at all, cannot be oxidized any more readily than the *meso*-form; unfortunately, no pure *d*-butanediol was available for a further examination of this point.

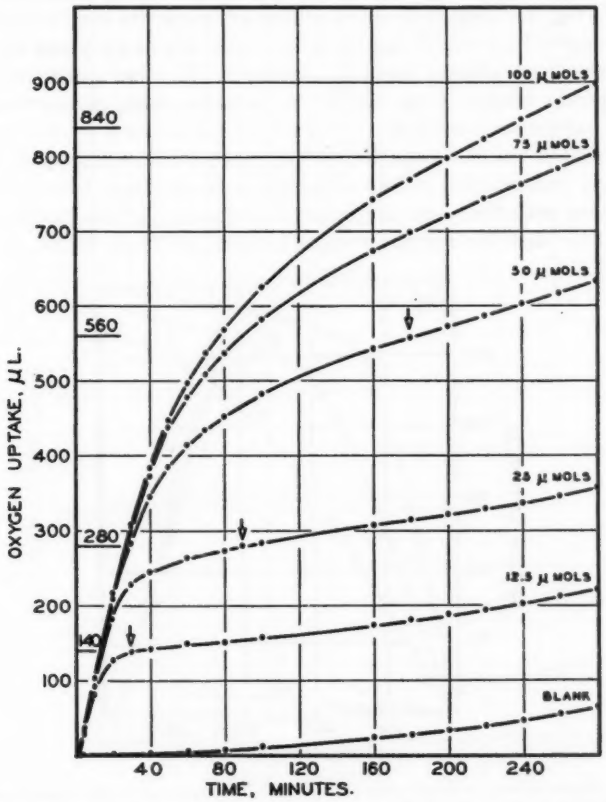


FIG. 4. *Oxygen uptake by Aerobacillus polymyxa with several different concentrations of l-butanediol. The arrows indicate the levels of oxygen consumption corresponding to oxidation to acetoin of the three lowest concentrations of substrate; the two highest concentrations were not oxidized to the acetoin stage during the period of the experiment.*

The oxygen uptake with *l*-butanediol seems to provide evidence that *A. polymyxa* is unable to carry the oxidation beyond the acetoin stage; however, with acetoin as a substrate a slow oxygen consumption is obtained (about double that of the autorespiration), which shows that this conclusion is not strictly correct.

The concentration of substrate has a profound influence on the course of butanediol oxidation. With 25 μmoles or less of *l*-butanediol one generally obtains curves that are almost linear to the point of substrate exhaustion and then break sharply to a rate approximating that of the autorespiration. Even in these cases, there is a slight progressive rate decrease during the period of substrate oxidation; the oxygen consumption is always at a maximum during the first 10 min. When the amount of substrate is increased to 50 or 100 μmoles, the character of the curves for oxygen uptake changes radically as shown in Fig. 4. The rate starts falling off markedly long before the substrate is completely oxidized, and there is no sign of a sharp break at the point where substrate exhaustion could be expected. In other words, the enzyme system is partly inhibited, the degree of inhibition being proportional to the amount of substrate provided. However, this inhibition is clearly not due to the concentration of butanediol *per se*, since the initial rate of oxidation is practically independent of the substrate concentration, being if anything slightly higher with the larger amounts of substrate. It thus appears reasonable to assume that the acetoin produced is the inhibitory factor.

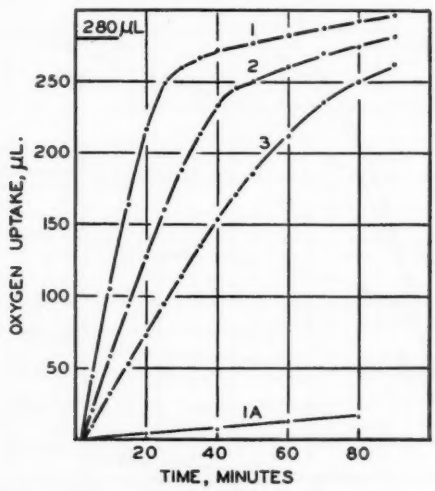


FIG. 5. *Oxygen uptake in the presence of 25 μmoles of l-butanediol by Aerobacillus polymyxa in three different cell concentrations. In all cases the same total volume was used. Curve 1 represents the oxygen uptake for the most concentrated cell suspension; Curve 2 for a suspension of half the cell concentration; and Curve 3, for a suspension of a quarter the cell concentration. Curve 1A represents the autorespiration for the highest cell concentration; the autorespiration for the two lower cell concentrations was too small to be shown.*

Such an inhibition is readily explicable as being due to competition between butanediol and acetoin for the enzyme, with consequent blocking of the oxidation by large amounts of acetoin. On the basis of this hypothesis, the degree of inhibition ought to be proportional to the ratio between the amounts of enzyme and acetoin present; it should thus be possible to increase inhibition by shifting the ratio in favour of acetoin. The ratio can thus be altered in two ways: either by decreasing the amount of cells (and hence of enzyme) or by adding acetoin to the cell suspension at the start of the oxidation. We have carried out both these experiments, with results that agree with our predictions.

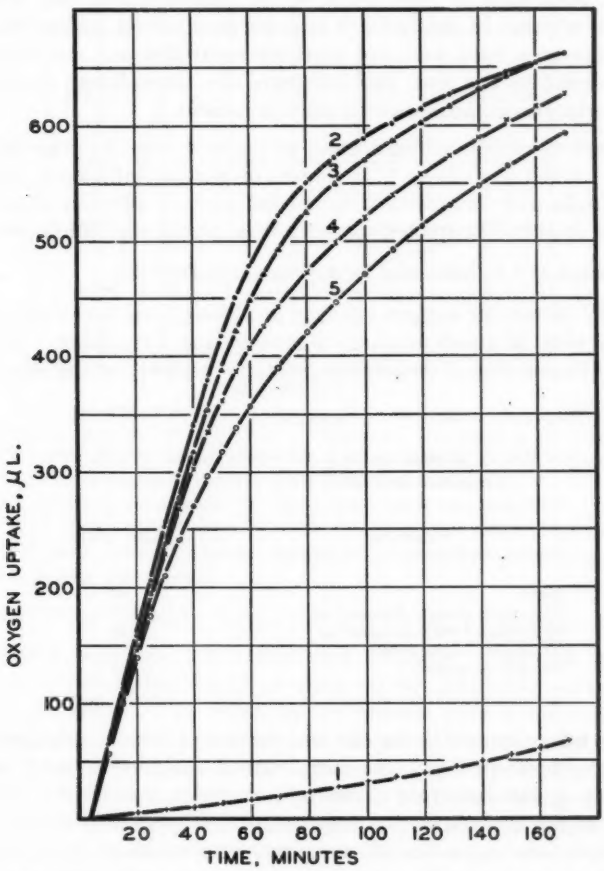


FIG. 6. *Oxygen uptakes by Aerobacillus polymyxa in the absence of substrate (Curve 1), in the presence of 50 μmoles of l-butanediol (Curve 2), 50 μmoles of l-butanediol + 6.25 μmoles of acetoin (Curve 3), 50 μmoles of l-butanediol + 25 μmoles of acetoin (Curve 4) and 50 μmoles of l-butanediol + 50 μmoles of acetoin (Curve 5).*

The oxidation of 25 μmoles of butanediol by cell suspensions of three different concentrations (cells in the ratio of 1 : 2 : 4) is shown in Fig. 5. The highest cell concentration gave a curve for oxygen uptake that showed very little inhibition, breaking sharply near the end-point; the lowest concentration showed characteristic inhibition; and the intermediate concentration showed a certain degree of inhibition, although the signs of a break near the end-point are still detectable. This experiment explains a previously observed anomaly; namely, that the shape of the curve for oxygen uptake can vary quite considerably with a given concentration of butanediol from one experiment to the next. This is seen, for example, in the curves for oxygen uptake with 25 μmoles of butanediol in Figs. 3 and 4; the former shows very little sign of inhibition, whereas in the latter it is quite pronounced. Since the amounts of cell suspension used were not carefully controlled and standardized from one experiment to the next, this difference can be explained logically on the basis of variations in the amount of enzyme present.

The effect of initially added acetoin on the oxidation of butanediol is shown in Fig. 6. Even as little as 6.25 μmoles of acetoin influences the curve for oxygen uptake with 50 μmoles of butanediol, and the addition of an equimolar amount of acetoin (50 μmoles) causes a very pronounced inhibition.

The Oxidation of 2,3-Butanediol by Aeromonas hydrophila

Table IV shows the oxygen uptakes in a three-hour period by *Aeromonas hydrophila* with 50 μmole amounts of acetoin and butanediol. With acetoin and pure *l*-butanediol, *A. hydrophila* is totally inactive; on the other hand, the

TABLE IV

TOTAL OXYGEN UPTAKE IN A THREE-HOUR PERIOD BY *Aeromonas hydrophila* WITH VARIOUS SUBSTRATES

Substrate	O_2 uptake, μlitres
None	99
50 μmoles *d–meso* butanediol	445
50 μmoles *l–meso* butanediol	435
50 μmoles *l*-butanediol	99
50 μmoles acetoin	109

butanediol mixtures containing the *meso*-isomer provoke a substantial oxygen uptake. As shown in Fig. 7, the initial rate of oxidation is rapid, but falls off increasingly as the substrate is oxidized, reaching a level not much greater than that of the autorespiration long before the theoretical oxygen consumption for oxidation to the acetoin stage has been reached. Judging from the close similarity between the curves for the *d–meso* and *l–meso* mixtures, it is probable that *A. hydrophila* is unable to oxidize the *d*-isomer, since one might expect a substantially greater oxygen uptake with the *d–meso* mixture were the *d*-isomer attacked.

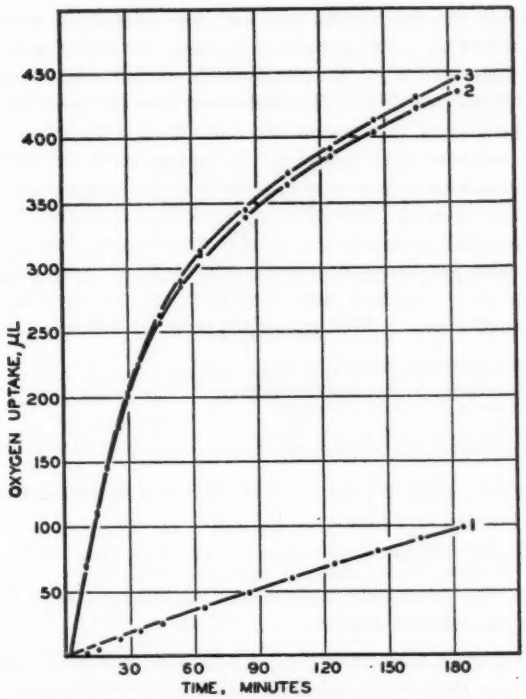


FIG. 7. *Oxygen uptake by Aeromonas hydrophila in the absence of substrate (Curve 1) and in the presence of 50 µmoles of l-butanediol (Curve 1), 50 µmoles of the mixture of l- and meso-butanediol (Curve 2) and 50 µmoles of the mixture of d- and meso-butanediol (Curve 3).*

Perhaps with smaller amounts of *meso*–butanediol a clear-cut oxidation to acetoin could have been obtained, as with *Aerobacillus polymyxa*, but the experiment was not attempted.

The Oxidation of Other Diols by Aerobacter aerogenes and Aerobacillus polymyxa

Since samples of several other diols were available, it seemed of interest to determine whether they could be oxidized by *Aerobacter aerogenes* and *Aerobacillus polymyxa*. The following compounds were examined: racemic 1,3-butanediol, 2-methyl-1,2-propanediol, racemic 1,2-propanediol, and 1,2-ethanediol. Only 1,2-propanediol provoked an oxygen uptake by *Aerobacter aerogenes* and *Aerobacillus polymyxa*, the rate in both cases being slower than with an equimolar amount of 2,3-butanediol. The oxygen uptake was the same with both organisms: approximately 0.25 moles per mole of substrate. Assuming only one optical isomer to have been attacked, this is equivalent to a removal of two hydrogen atoms, which would lead to the formation either of acetol (attack on the secondary alcohol group) or lactic aldehyde (attack on the primary alcohol group).

In an attempt to identify the product, the experiment was repeated on a larger scale. Dense washed cell suspensions of *Aerobacter aerogenes* and *Aerobacillus polymyxa* were prepared, and to 100 ml. of each was added 0.6 gm. of 1,2-propanediol. The mixtures were aerated at 30° C. for five hours. At the end of this period the cells were removed by centrifugation and the supernatants were tested for the presence of the possible products. Both supernatants showed strong reduction of Fehling's solution in the cold, a positive reaction with Tollens' reagent, and a negative Schiff's reaction. They were then ether extracted, and following evaporation the ether extracts were treated with semicarbazide hydrochloride. In both cases a crystalline semicarbazone was obtained and identified by the melting point as the semicarbazone of acetol. Melting points were as follows (corrected):

(1) Semicarbazone from *Aerobacillus* supernatant	199° C.
(2) Semicarbazone from *Aerobacter* supernatant*	193° C.
(3) Semicarbazone of synthetic acetol**	198° C.
(4) Mixed melting point of (1) and (3)	198° C.
(5) Mixed melting point of (2) and (3)	194° C.

The results thus showed that 1,2-propanediol was oxidized by both organisms according to the equation:

$$CH_3 . CHOH . CH_2OH + \tfrac{1}{2}O_2 \longrightarrow CH_3 . CO . CH_2OH + H_2O.$$

This oxidation has previously been demonstrated by Kling (6) and Visser't Hooft (10) for *Acetobacter* species.

Although this comparative study is far from complete, it suggests that the conditions for the dehydrogenation of diols by *Aerobacter aerogenes* and *Aerobacillus polymyxa* are sharply limited (Table V). These organisms are unable to dehydrogenate primary alcoholic groups (no attack on 2-methyl-1,2-propanediol or 1,2-ethanediol), and even on secondary alcoholic groups their activity is controlled by structural factors. The immunity of the secondary

TABLE V

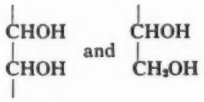
SUMMARY OF THE ACTION OF *Aerobacter aerogenes* AND *Aerobacillus polymyxa* ON DIOLS

1,3-Butanediol	2,3-Butanediol	2-Methyl-1,2-propanediol	1,2-Propanediol	1,2-Ethanediol
CH_3 | $CHOH$ | CH_2 | CH_2OH	CH_3 | $CHOH$ | $CHOH$ | CH_3	CH_3 CH_3 \ / $C—OH$ | CH_2OH	CH_3 | $CHOH$ | CH_2OH	CH_2OH | CH_2OH
Not attacked	Oxidized to acetoin or further. Specificity of attack on different isomers.	Not attacked	Oxidized to acetol. Specificity of attack on different isomers.	Not attacked

* *This sample appeared quite impure; owing to the small amount obtained, it could be recrystallized only once.*

** *The synthetic acetol was prepared according to the method of Levene and Walti (7).*

alcoholic group in 1,3-butanediol, as contrasted with the dehydrogenation of secondary alcoholic groups in 2,3-butanediol and 1,2-propanediol, implies that *Aerobacter aerogenes* and *Aerobacillus polymyxa* can attack this group only when it is adjacent to another secondary alcoholic group or to a primary one:



Further work will be needed to confirm the validity of this hypothesis.

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